Are sperm characters related to genetic diversity in the bluethroat (*Luscinia svecica*)?

Master of Science Thesis in Ecology and Evolution

By

Kristine Dobbe

Natural History Museum

University of Oslo

2014

Are sperm characters related to genetic diversity in the bluethroat (*Luscinia svecica*)?

Master of Science Thesis in Ecology and Evolution

Kristine Dobbe

Natural History Museum

University of Oslo

2014

2014

Are sperm characters related to genetic diversity in the bluethroat (*Luscinia svecica*)?

Kristine Dobbe

http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo

Forord

Først og fremst, en stor takk til mine veiledere, professor Arild Johnsen og Terje Laskemoen. Jeg har vært veldig heldig syns jeg selv, og setter stor pris på all hjelp jeg har fått. I tillegg er jeg veldig glad for at jeg fikk denne muligheten til å skrive oppgaven her hos dere på Naturhistorisk museum. Oppgaven har hatt elementer som feltarbeid, labarbeid (både DNAlab og spermlab), statistiske analyser, databehandlingsprogram, ja, rett og slett en god blanding av alt mulig gøy.

En stor takk til alle dere i Frankrike for feltarbeidet der: Marie-Christine Eybert, Laurent Godet, Jérôme Fournier, Patrick Bonnet og Matthieu Marquet. Det var helt supert!

Takk går selvfølgelig også til alle som har gitt meg hjelp til både smått og stort i denne tiden jeg har jobbet med oppgaven. Lars Erik Johannessen for feltarbeid (både Frankrike og Heimdalen), for super løsning av «Excel-problematikk» og for data-tilgang av databasen. Gunnhild Martinsen for god opplæring både på lab og i GeneMapper, men også hjelp til labarbeid og analysering av spermfart. Silje Hogner for «reruns» og tilgang til materialet. Jostein Gohli og Trond Øigarden for god hjelp i felt (Heimdalen). Even Stensrud for feltarbeid (Heimdalen) og opplæringen av preparering av spermprøver for fotografering. I tillegg takk til Becky for hjelp til omregning av data. Og selvfølgelig for at dere er en super duper gjeng!

For all støtte, «pep-talk» og hyggelig samvær under hele prosessen (i tilfeldig rekkefølge), Trude, Maria, Lena, Even, Becky, Anna, Eilen, Silje, Are og Jan Erik. Familien min vil jeg også takke, mamma og pappa, storesøster og storebror, tante Kari og tante Tullemor. Takk for at dere har støttet meg og heia på meg.

Kristine Dobbe

13. mai 2014, Oslo

Abstract

Heterozygosity is often shown to have a positive relationship with fitness-related characters. Sperm cells are fitness-related characters that show great variation among species, population's males and even within individual males. I will investigate relationships between heterozygosity and sperm characters in two bluethroat (*Luscinia svecica*) subspecies, *L.s.svecica* and *L.s.namnetum*. I have three main aims: 1) test whether previously found differences between the two subspecies in genetics and sperm characters will be upheld and/or strengthened. Further, I will add two aspects of sperm behavior that has not been studied in these subspecies before, namely sperm velocity and proportion of motile sperm cells, 2) test the hypothesis that individual heterozygosity positively influences the expression of fitness-related sperm characteristics, and 3) investigate relationships among sperm characters at the level of the individual males, testing whether sperm morphology is related to sperm behavior. I used microsatellite genotyping (25 loci) to calculate the genetic diversity, and measured the sperm morphology (sperm length) and behavior (i.e. proportion of motile sperm cells and sperm velocity). There was little evidence for relationships between heterozygosity and any of the sperm characters, except for a significant relationship between heterozygosity and velocity when including data from all males (i.e. also males with < 10 motile sperm filmed). In support of previous studies, I found a strong genetic differentiation between the two subspecies, as well as differences in individual heterozygosity, in body size and red border width. Furthermore, the two subspecies differed strongly in sperm morphology, proportion of motile sperm and the within-male coefficient of variation in sperm length. There were no significant relationships between sperm morphology and aspects of sperm behavior. This study suggests a need for further prospects with larger sample sizes before a firm conclusion can be reached regarding the relationship between heterozygosity and sperm characters in the bluethroat.

Table of contents

Introduction

Heterozygosity (i.e. individually genetic diversity) often shows positive relationships with fitness-related characters (Kempenaers 2007, Olano-Marin *et al.* 2011a, Olano-Marin *et al.* 2011b). Inbreeding will reduce the heterozygosity in the genome, which may increase the expression of deleterious alleles and lower the fitness of individuals (Charlesworth and Willis 2009). Examples of fitness-related characters that have shown positive relationships with heterozygosity include body size, plumage and antlers (Kempenaers 2007). A study on iberian red deer (*Cervus elaphus hispanicus*) found that males with small antlers had lower levels of heterozygosity than those with bigger antlers (Perez-Gonzalez *et al.* 2010). Another recent study, on crested newt (*Triturus cristatus*), found that heterozygosity significantly predicted the males' body size, and that male body size significantly predicted crest height (Herdegen *et al.* 2013). Additionally, Herdegen *et al.* (2013) found that heterozygosity significantly increased with age, which indicate that it had a positive effect on survival. Furthermore, a study on blue tits (*Cyanistes caeruleus*) found a positive relationship between male heterozygosity and crown feather colour (chroma) (Foerster *et al.* 2003). Heterozygosity-fitness correlation may be mediated by a general genetic effect, affecting the individual's overall physical health (condition/state/viability), or local genetic effects, influencing traits more directly (Møller 1997, Hansson and Westerberg 2002, Kempenaers 2007). As a possible example of the former, heterozygosity may affect the developmental stability, i.e. the ability of individuals to undergo stable development of their phenotype under certain environmental conditions (Møller 1997).

Sperm cells show great variation between species, between populations within species, between males and within a male (Cohen 1977, Parker 1998). Sperm length has been shown to correlate with sperm competition (i.e. the competition between the sperm cells of different males to fertilize the egg from a female) in several taxa, e.g. mammals (e.g. Hosken 1997, Breed and Taylor 2000, Gage and Freckleton 2003), insects (e.g. Gage 1994, Morrow and Gage 2000), fish (e.g. Stockley *et al.* 1997, Balshine *et al.* 2001) and birds (e.g. Immler and Birkhead 2007, Kleven *et al.* 2009, Lüpold *et al.* 2009b). Recent studies of passerine species show a clear negative correlation between intraspecific variation in total sperm length (both within and between males) and the risk of sperm competition (Kleven *et al.* 2008, Lifjeld *et al.* 2010). Laskemoen *et al.* (2007) demonstrated a significant variation in sperm length at the intraspecific level in the bluethroat (*Luscinia svecica*) and the willow warbler (*Phylloscopus*

trochilus), and that the sperm are more variable between males than within males. The degree of individual heterozygosity may influence sperm characteristics, such as the cell's uniformity, and sperm length and motility. A study on wild rabbits (*Oryctolagus cuniculus*) showed that loss of heterozygosity had a negative effect on the production of normal sperm, both between and within populations (Gage *et al.* 2006). Another study on multiple mammal species showed that species with higher level of homozygosity had reduced sperm quality, both in terms of sperm abnormality and motility (Fitzpatrick and Evans 2009). To my knowledge, no studies of the relationship between individual heterozygosity and sperm characters have been conducted in passerine birds.

The bluethroat is a socially monogamous passerine, with a relatively high rate of extra-pair paternity (EPP) (Questiau *et al.* 1999, Johnsen and Lifjeld 2003). For example, a study by Questiau *et al.* (1999) on the subspecies *L. s. namnetum* showed that 41.9 per cent of the offspring were sired by extra-pair males, and a study by Johnsen and Lifjeld (2003) on *L. s. svecica* showed that 7-33 per cent of the offspring were sired by extra-pair males, over a 10-year period. This high rate of EPP means that bluethroat males experience a high risk of sperm competition. The males have a colorful throat, which the female generally lack. It has been shown that this sexual ornament is important for mate choice in bluethroats (Johnsen and Lifjeld 1995, Johnsen *et al.* 1998a, Johnsen *et al.* 1998b). The species consists of about 10 subspecies (Cramp 1988), some that live in mountain habitats, and others in low land habitats. In this thesis, I will study relationships between individual heterozygosity and sperm characteristics, using two of the bluethroat subspecies, *L. s. svecica* (hereafter referred to as *svecica*) and *L. s. namnetum* (hereafter referred to as *namnetum*), as study systems. The study populations breed in very different habitats, *svecica* in a sub-alpine mountain valley in Norway and *namnetum* in salt marshes and reed beds at sea level in France. The coloration of these two subspecies has already been studied, and there are clear differences between them, both in the color of the throat spot (chestnut/white) and in the chroma of the blue feathers (Johnsen *et al.* 2006). The two subspecies also differ in body size (Johnsen *et al.* 2006, Hogner *et al.* 2013). A recent study found a significant difference in sperm head length between the two subspecies, where *svecica* had the longest sperm heads and tended to have the longest sperm cells (Hogner *et al.* 2013). The main reasons for choosing to study these two subspecies are that they are genetically distinct (Questiau *et al.* 1998, Johnsen *et al.* 2006), and geographically isolated. Furthermore, the two subspecies differ greatly in population size and hence degree of genetic isolation: *svecica* is widely distributed over most

of the northern Palearctic, while *namnetum* only is located at scattered localities along the coast of Brittany in France (Johnsen *et al.* 2006) (see Figure 1). This may cause *namnetum* to be a more inbred, less genetically variable population, potentially increasing the likelihood of detecting relationships between variation in heterozygosity and sperm characters.

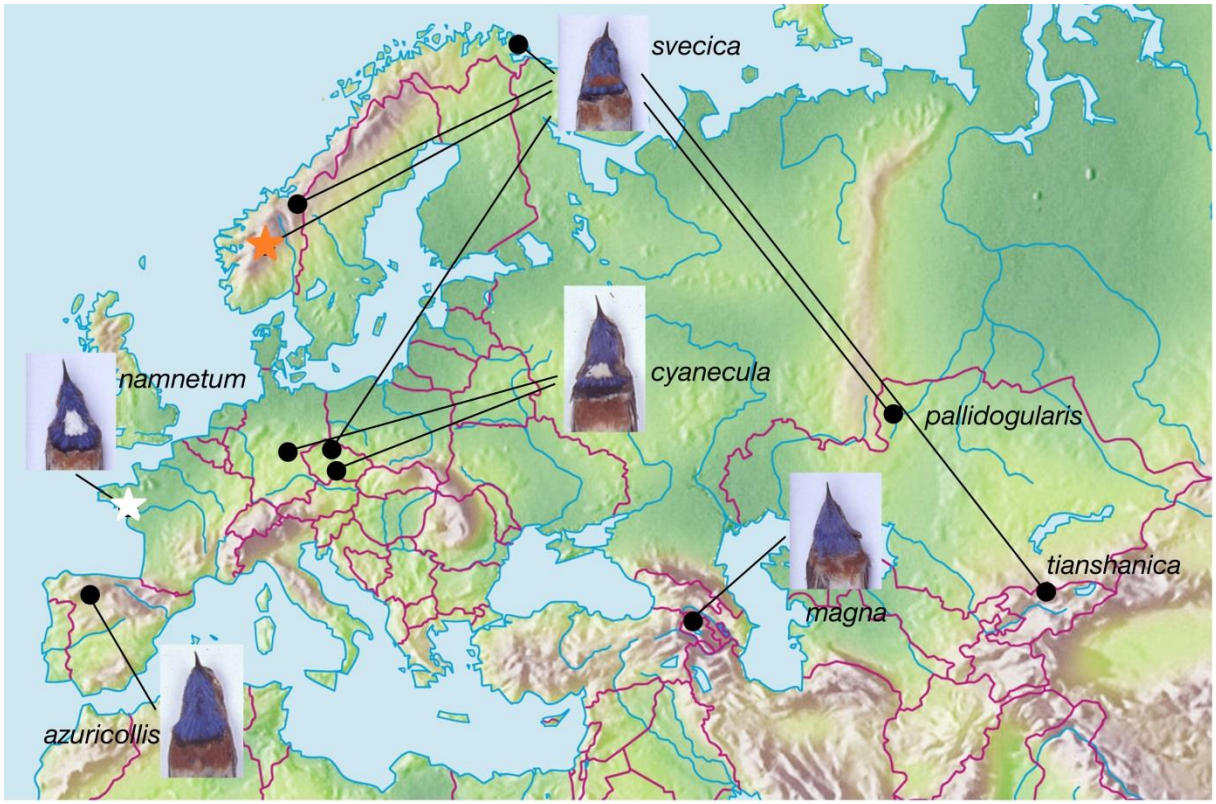

Figure 1: Map illustrating the distribution of sampling sites for seven subspecies of the bluethroat in the former study by Johnsen et al. (2006). Sampling sites for the two subspecies included in the present study are marked with an orange (*L. s. svecica*) and a white star (*L. s. namnetum*).

I have three main aims with this thesis. First, I will test whether previously found differences between the two subspecies in genetics and sperm characters will be upheld and/or strengthened with a larger sample size and higher number of microsatellite markers. Also, I will add two aspects of sperm behavior that has not been studied in these subspecies before, namely sperm velocity and proportion of motile sperm cells. Second, I will test the hypothesis that individual heterozygosity positively influences the expression of fitness-related sperm characteristics. I predict that the level of heterozygosity will be positively correlated with sperm characters, such as cell length, uniformity, velocity and proportion of motile sperm cells. In addition, one might expect a greater effect of genetic variability in *namnetum* than in *svecica* (see above). Finally, I will investigate relationships among sperm characters at the level of the individual males, testing whether sperm morphology is related to sperm behavior.

Material and methods

Study sites and species

I have studied two geographically isolated bluethroat populations, *svecica*, in Norway (Øvre Heimdalen, Øystre Sildre, Oppland) (61°25'N, 8°52'E) and *namnetum*, in France (Guèrande /Brière) (47°17'N, -2°28'E/ 47°21'N, -2°12'E). Heimdalen is a mountain habitat and lies east of the Jotunheimen Mountains (Figure 2a). The valley is situated about 1100m above sea level, and has sub-alpine vegetation. Guèrande /Brière are salt marsh /reed bed habitats and are located on the coast of Brittany, France (Figure 2b).

The bluethroat is a relatively small (about 16g) passerine bird in the Muscicapidae family. It is a migratory bird and males arrive first to the breeding sites (about a week before females) to establish territories. Soon after pair formation the female builds her nest in the vegetation on the ground, and lays 5-7 eggs which she incubates for 13-15 days (without male help). Both of the parents (female and the social male) feed the nestlings, until the chicks leave the nest (10-14 days after they hatch). The main differences between the two subspecies, *svecica* and *namnetum*, are in the coloration of the male's throat spot (chestnut in *svecica*, white in *namnetum*), body size (*svecica* being larger than *namnetum*) and the genetic constitution (Johnsen *et al.* 2006, Hogner *et al.* 2013) (Figure 3).


a)

b)

Figure 2: Photos representing the habitat of *svecica* (a) and *namnetum* (b). Photos: Arild Johnsen (a) and Kristine Dobbe (b).

Figure 3: Males of the two subspecies, showing the different throat-spot color, white and chestnut-brown on *namnetum* and *svecica*, respectively. Photos: Kristine Dobbe

Field-sampling

The birds were captured with mist nets and playback (both male and female song) or clap nets using mealworm as bait. Males were captured in their territories in the breeding season, April (France) and June (Norway) 2012. When the birds were captured, I took multiple measurements: length of tarsus (to nearest 0.1mm) using a slide caliper, wing length (to nearest 0.5 mm) using a wing ruler, body mass (to nearest 0.1 g) using a pesola 50g spring balance, width of the red border (to nearest 1 mm) using a slide caliper, in addition taking blood sample and sperm sample. The birds were also aged (second year or older) by inspecting the wing feathers (the coverts) (Svensson 1992), and photos were taken of each bird. The blood samples were taken by puncturing the brachial vein on the wing, and then

blood was captured by a capillary tube, and then stored in 2 ml Sarstedt tubes with1ml absolute ethanol for later genetic analysis. The sperm samples (ejaculates) were sampled in a cloacal massage technique (Wolfson 1952), collected by a capillary tube and instantly diluted in an Eppendorf tube (about 30µl) containing pre-heated DMEM, for motility measures (more details in the motility paragraph below). The remaining ejaculate was stored in 5 per cent formalin (PBS) solution for later sperm morphology measurements. All of the captured birds were marked with a unique metal ring for identification. After processing the birds, they were all released back in the wild.

From *svecica*, I caught 44 bluethroats (31 males and 13 females), and from *namnetum* I caught 26 bluethroats (22 males and 4 females) in the season of 2012. To get more data, and to increase the power for the statistical analyses, I included some birds sampled in previous years as well. From *svecica* I added birds caught from the years 2007-2010 (28 males), and from *namnetum* I added birds caught in 2011 (16 males). However, some of the measures were missing of the birds from the previous years, e.g. genotypes, sperm characters or morphological measurements. In the genetic analysis, I also included the females (n = 17) in order to increase the power of the analyses.

The length of the tarsus is an indication of the skeleton size of the bird. Therefore, I used tarsus length as the measure of the body size of the birds, since this is more fixed measure than body mass, which may vary throughout the day and season. In addition, I used wing length as a measure of size, even if it is more exposed to wear and tear.

Analyses

DNA extraction and microsatellites

The DNA was extracted from blood using Omega Bio-Tek (E-Z 96 Blood DNA Kit (D1199-01)), using the manufacture's protocol. 37 microsatellites were amplified by polymerase chain reaction (PCR) (GeneAmp® PCR System 9700 (Applied Biosystems)), and ran on an ABI Prism® 3130XL Genetic analyzer (Applied Biosystems) using fluorescently labeled primers. Allele sizes were determined using ABI Prism® GeneMapper™ Software version 4.0 (Applied Biosystems).

Each DNA extract was diluted 1:3. The 37 markers used are originally pied flycatcher (*Ficedula hypoleuca*) markers (Leder *et al.* 2008), but also optimized for bluethroat. They were sorted in five panels (1-5), and run using multiplex PCRs (see Table 2 in appendix for details). The primer-mix consisted of various volumes of forward and reverse primers from all markers in each panel (see details in Table 2). To each of the PCR I added 5 µl 2x Qiagen Multiplex Master Mix (QIAGEN), 1 µl primer-mix, 3 µl Rnase-free water and 1 µl DNA extract (in total 10 µl volume for each sample). For all panels, the following PCR program was used: denaturizing; 95°C for 15 min, 94°C for 30 sec, 56°C or 59°C annealing temperature for 1:30 sec, for panels 2-5 and panel 1 respectively, elongation; 72°C for 1 min, this profile were repeated for 34 more cycles before a final elongation step of 60°C for 15 min. To confirm amplification success I used 3 µl of the PCR product to test it on an electrophoresis agarose gel (1 per cent). After PCR, the samples were diluted 1:99 before preparation for the ABI. For panel 1-4 2 µl PCR products was added to the ABI plate, for panel 5 only 1µl PCR product was added, both from the diluted PCR product. The same amount, 9.5 µl HiDi and 0.5 µl Liz 600 were added to each sample in all the panels for running in the ABI.

I performed Hardy-Weinberg (HW) test and a null-allele test in the software Cervus version 3.0.3 (see Appendix Table 3). A limit for F(null) at 0.10 was set, and all the markers that showed a higher value were excluded from further analysis, because null-alleles lead to erroneous heterozygosity estimates. Additionally, I performed linkage disequilibrium test between the different markers in Fstat version 2.9.3 (Goudet 1995). This showed a linkage between the markers ZF-C59 and EST16, so EST16 was excluded (due to more data on ZF-C59). The same result was shown between Fh405 and Fh224, but Fh224 had already been excluded by the null-allele test. Marker Fh465 did not give any results. This leads to a total of 25 markers in the further analysis.

I used Cervus for calculating the locus characteristics (see Appendix Table 3). I also used Fstat to calculate population-specific allelic richness, which refers to the total number of alleles per locus in the populations, adjusted for the number of individuals typed at each locus (see Appendix Table 4). To compare allelic richness in the two subspecies, I ran a paired t-test in SPSS. Overall genetic differentiation was estimated by the F_{ST} index (Weir and Cockerham 1984) using Fstat. The sample in the genetic analyses consisted of 75 males (*svecica*; n = 40,

namnetum; n = 35), and 17 females (*svecica*; n = 13, *namnetum*; n = 4), leading to a total sample size of 92 individuals.

Heterozygosity

Not all markers are represented for all the individuals, therefore I calculated standardized heterozygosity (SH) by dividing the proportion of heterozygous loci for an individual by the mean observed heterozygosity for all loci typed for that individual (Coltman *et al.* 1999). Pearson correlation test show that H and SH correlate strongly ($r = 0.998$, $p<0.001$, $n=92$). I will therefore use SH (with 25 markers) in the further analysis.

Sperm analyses

Morphology

Approximately 10-15µl of diluted sperm was spread out on a microscope slide with a pipette. Then I let it air-dry overnight. When dry it formed crystals that needed to be washed off, using distilled water. The slide was again allowed to dry for at least one hour. From each male I took pictures of 30 normal sperm cells, using a Leica DFC420 camera mounted on a Leica DM6000 B digital light microscope to obtain digital images at magnification of 160x, and measured the length with specialized image analysis software Leica Application suite version 4.1. The sperm cells are divided in three components; head, midpiece and tail (see Figure 4), which were measured separately. The total length of the sperm cell was calculated by adding the length of these three components. In addition, I calculated the midpiece/total sperm length ratio. For the males that were caught the earlier years, only 10 sperm cells were measured per male, and these sperm cells were measured by an another person (Terje Laskemoen). There were 43 males with 30 sperm cells measured, and 40 males with 10 sperm cells measured, which leads to a total of 83 males with morphometric measurements (*svecica*; n = 53, *namnetum*; n = 30).

I calculated within-male coefficient of variation of total sperm length, (CVwm (CV = SD/mean*100)). Additionally, I calculated the between-male coefficient of variation of the total sperm length (CVbm) for the two subspecies. Since coefficient of variation estimates can be affected by sample size (e.g. Laskemoen *et al.* 2007), I adjusted the CVbm values using the

following equation (CV+ (1/4n)). Hereafter all CVbm values reported are adjusted for sample size.

Figure 4: Microscope image of bluethroat sperm, illustrating the three components of the sperm cell; head, midpiece and tail. Photo: Kristine Dobbe

Motility

Immediately after the sperm were collected (in field, see above), the samples were diluted in pre-heated Dulbecco's Eagel Medium (DMEM) set to 40ºC. Then 3–5 ml of the diluted sperm was placed in a pre-heated microscopy counting chamber (depth 20 mm; Leja Products BV, Nieuw-Vennep, the Netherlands) and mounted on a MiniTherm stage warmer (Hamilton Thorne Bioscciences, Beverly, MA) maintained at a constant temperature of 40ºC. Sperm movement was then recorded using a phase contrast microscope (model CX41, Olympus, Japan) with a connected digital video camera (model HDR-HC1C, Sony, Tokyo, Japan) (Laskemoen *et al.* 2013b). Each of the sperm samples were recorded for about 30 seconds, and 6 frames were used to optimize the recording of the sperm cells.

For measuring the motility of the sperm cells, a computer - assisted sperm analysis (HTM-CEROS II Sperm Analyzer; Hamilton Thorne Research, Beverly, MA) was used. The sperm analyzer was set at a frame rate of 50 Hz and 25 frames (i.e., sperm cells were tracked for 0.5 second). Each analysis was visually examined and cell detection parameters were adjusted using the two interactive quality control plots as well as directly from visual examination of

each recording. Three estimates of sperm velocity were recorded: straight line velocity (VSL; i.e. velocity on a straight line between start and end points of the sperm track), average path velocity (VAP; i.e. average velocity over the sperm track), and curvilinear velocity (VCL; i.e. velocity over the actual sperm track). To remove the potential effect of drift in the chamber, sperm cells with VAP less than 10 μm s^{-1} and VSL less than 5 μm s^{-1} were counted as static and excluded from the swimming speed analysis. Proportion of motile sperm was also calculated. According to earlier sperm motility studies, these three different tracker methods inter-correlate strongly (Kleven *et al.* 2009, Rowe *et al.* 2013, Laskemoen *et al.* 2013b), which leads to similar result. Since the curvilinear velocity (VCL) measures the actual sperm track, I will only use this in the further analysis, referring to it as sperm velocity. I performed two sets of analysis; 1) including only males with a minimum of 10 motile sperm cells filmed, 2) including males with a minimum of 4 motile sperm cells per male. The total number of males in the analysis from set 1) was 36, with a range from 10-252 mobile sperm cells, divided in the two subspecies, *svecica*: n = 24, and *namnetum*: n = 12. The total number of males in the analysis from set 2) was 43, with a range from 4-252 motile sperm cells (seven males with < 10 sperm), divided in the two subspecies, *svecica*: n = 29 and *namnetum*: n = 14.

Statistical analyses

I ran the statistical analysis in IBM SPSS v 21.0 and STATISTICA version 7.0. Kolmogorov-Smirnov and Shapiro-Wilk test were used for testing for normality. Only the variables that show no significant departure from normality in both of the tests were treated as normally distributed. I used parametric Pearson correlation and Student's t –tests on the normally distributed data, and for the non-normal data, I used non-parametric equivalents tests (Spearman correlation and Mann-Whitney U test). General linear models (GLM) were used on normally distributed response variables, including "subspecies" as a fixed factor, and the interaction between the "subspecies" and the independent variable. Predictor variables were removed by backward stepwise exclusion when $p > 0.10$.

Results

Between-population differences in genotypes and phenotypes

Genetic differentiation

The two populations were genetically differentiated, with an overall F_{ST} value of 0.066 (p = 0.0002). The two populations differed in the degree of genetic variability with *namnetum* showing lower allelic richness across markers ($t_{24} = 6.61$, $p < 0.001$) (see Appendix Table 4). Indeed, a test at the individual level showed that birds belonging to *svecica* were significantly more heterozygous than those from *namnetum* ($t_{90} = 5.9$, $p < 0.001$) (see Figure 5).

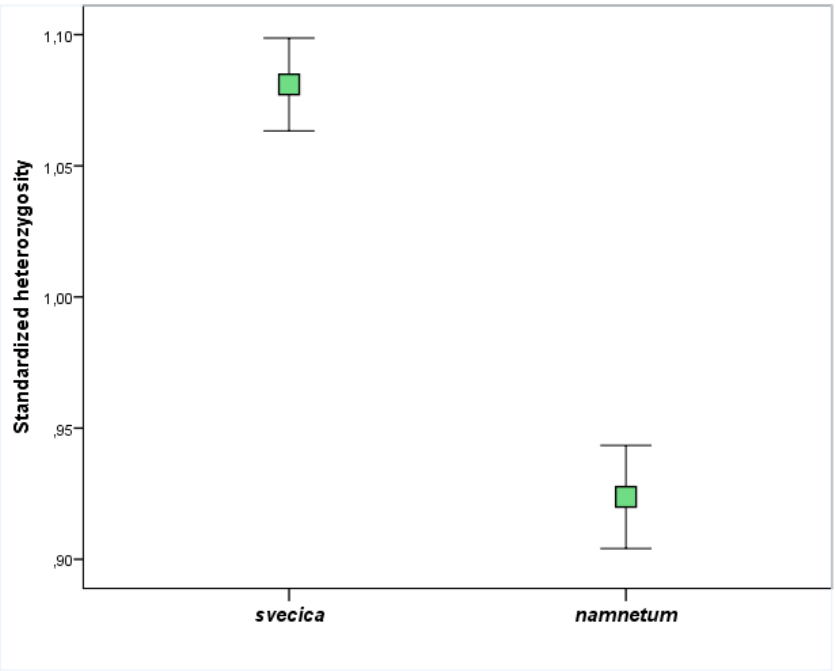

Figure 5: Average heterozygosity (± SE) in the two subspecies.

Morphological differentiation

Birds of the *svecica* subspecies were significantly bigger than *namnetum* by body size (measured in tarsus length and wing length) (see Table 1). The red border was significantly wider in *namnetum* than *svecica* (see Table 1), and the variance in the red border width was higher in *svecica* than in *namnetum* (Levene's test, $F_{1,52} = 8.24$, $p = 0.006$). I did not find any difference in the width of the red border between old and young males when testing within subspecies (*svecica*: Mann-Whitney U test, $n_{young} = 12$, $n_{old} = 20$, $U = 88.5$, $p = 0.21$;

namnetum: Mann-Whitney U test, $n_{young} = 6$, $n_{old} = 16$, $U = 39$, $p = 0.37$). There was no difference in age distribution between the two subspecies (Fisher's exact test, $p = 1$).

Sperm differences

I found a significant difference in total sperm length between *svecica* and *namnetum* (Table 1). Additionally, the head length and midpiece length differed significantly between the two subspecies (Table 1). Sperm velocity was not significantly different in the two subspecies, but there was a significant difference in the proportion of motile sperm cells, where *namnetum* had the highest proportion (Table 1). The mean CVbm was remarkably similar in the two subspecies (Table 1). However, the mean CVwm in sperm length was significantly higher in *namnetum* (Table 1).

Table 1: Summary of the different sperm components and body biometrics measures of the two subspecies. CVwm and CVbm are the mean coefficient of variation in sperm total length, within males and between males, respectively. All of the individuals are males, and sample sizes are indicated with n.

Variables	***svecica*** **mean ± SD**	***namnetum*** **mean ± SD**	**Test statistics**	**p**
Sperm morphology	n = 53	n = 30		
Head length	19.78 ± 0.72	18.71 ± 0.65	$t = 6.68$	**p < 0.001**
Midpiece length	176.06 ± 7.54	171.49 ± 5.81	$t = 2.84$	**p = 0.006**
Tail length	15.92 ± 4.58	15.92 ± 3.7	$U = 766$	p = 0.78
Total length	211.76 ± 5.60	206.12 ± 5.39	$t = 4.47$	**p < 0.001**
Range in total length	201.04-227.95	195.74-214.93		
Midpiece length/total length ratio	0.83 ± 0.02	0.83 ± 0.02	$U = 764$	p = 0.77
CVwm	1.53 ± 0.56	1.75 ± 0.53	$U = 567.5$	**p = 0.03**
CVbm	2.65	2.62		
Sperm motility	n = 29	n = 14		
VCL	151.68 ± 22.98	140.39 ± 32.62	$t = 1.31$	p = 0.10
Proportion motile	0.58 ± 0.18	0.74 ± 0.15	$t = -2.94$	**p = 0.005**
Body morphology	n = 31/32/32[1]	n = 35/36/22[1]		
Tarsus length	30.25 ± 0.67	27.60 ± 0.72	$t = 14.10$	**p < 0.001**
Wing length	75.77 ± 2.19	68.38 ± 1.38	$t = 18.05$	**p < 0.001**
Red border	6.72 ± 2.45	7.41 ± 1.14	$U = 240$	**p = 0.04**

[1]Sample sizes for tarsus length/wing length /red border, respectively. P-value < 0.05 in bold.

Relationships between genetic diversity and sperm characters

I found no significant relationships between heterozygosity and total sperm length (GLM: $F_{1,60} = 0.18$, $p = 0.67$) or any of the other sperm morphometric variables (all $p > 0.14$). Furthermore, heterozygosity was not related to midpiece/total length ratio (*svecica*: $n = 34$, $r_s = 0.01$, $p = 0.94$, *namnetum*: $n = 27$, $r_s = -0.04$, $p = 0.83$) or CVwm (*svecica*: $n = 34$, $r_s = -0.17$, $p = 0.34$, *namnetum*: $n = 27$, $r_s = 0.01$, $p = 0.98$).

There was no relationship between heterozygosity and velocity (GLM: $F_{1,35} = 0.58$, $p = 0.45$) when including only males with 10 or more motile sperm. However, when including all males, there were a significantly positive correlation between heterozygosity and velocity (GLM: $F_{1,42} = 5.07$, $p = 0.03$) (Figure 6). In addition, there was a tendency for the proportion motile sperm to be positively correlated with heterozygosity (GLM: $F_{1,35} = 3.08$, $p = 0.09$), when including only males with 10 or more motile sperm cells (result identical when including all males, $p = 0.09$).

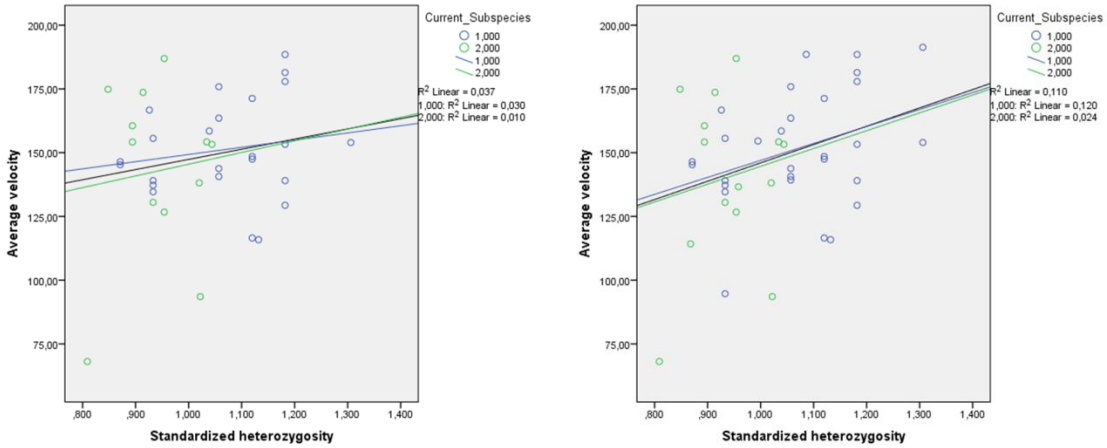

Figure 6: Scatterplots illustrating the heterozygosity against velocity, with and without a cut-off at 10 motile sperm cells, respectively. 1/blue represents *svecica* and 2/green *namnetum*.

Relationship between genetic diversity and morphological traits

I found no correlation between heterozygosity, and tarsus length (GLM: $F_{1,79} = 1.25$, $p = 0.27$) or wing length (GLM: $F_{1,80} = 0.07$, $p = 0.80$). Additionally, there were no relationship between heterozygosity and red border width in either of the two subspecies (*svecica*: $n = 31$, $r_s = 0.04$, $p = 0.81$, *namnetum*: $n = 22$, $r_s = -0.32$, $p = 0.14$).

Relationships among sperm characters

Correlations between sperm morphology and motility

Total sperm length did not predict sperm velocity (GLM: $F_{1,33} = 0.004$, $p = 0.95$) nor did any of the other sperm morphometric variables (all $p > 0.49$). There was no relationship between the midpiece/total sperm length ratio and sperm velocity (*svecica*: $n = 23$, $r_s = 0.20$, $p = 0.37$, *namnetum*: $n = 11$, $r_s = -0.03$, $p = 0.93$) or between CVwm and velocity in either of the two subspecies (*svecica*: $n = 23$, $r_s = -0.02$, $p = 0.91$, *namnetum*: $n = 11$, $r_s = -0.28$, $p = 0.40$). Furthermore, there were no relationships between total sperm length and the proportion of motile sperm cells (GLM: $F_{1,33} = 2.61$, $p = 0.12$) or any of the other morphometric variables (all $p > 0.57$). Likewise, there was no relationship between the CVwm and the proportion of motile sperm cells (*svecica*: $n = 23$, $r_s = 0.01$, $p = 0.95$, *namnetum*: $n = 11$, $r_s = 0.45$, $p = 0.16$) or between the midpiece/total length ratio and the proportion of motile sperm cells (*svecica*: $n = 23$, $r_s = -0.28$, $p = 0.19$, *namnetum*: $n = 11$, $r_s = 0.45$, $p = 0.16$).

Correlations among the different sperm morphology characters

I found a significant negative correlation between CVwm and midpiece length in *namnetum* ($n = 30$, $r_s = -0.45$, $p = 0.01$), but not in *svecica* ($n = 53$, $r_s = -0.13$, $p = 0.35$). Additionally, there was a significantly negative correlation between CVwm and the midpiece/total sperm length ratio in svecica ($n = 53$, $r_s = -0.31$, $p = 0.02$) and a similar trend in *namnetum* ($n = 30$, $r_s = -0.33$, $p = 0.07$).

Other correlations with sperm characters

In the initial model, I found an interaction effect between tarsus and subspecies bordering on significance (GLM: $F_{1,35} = 3.04$, $p = 0.09$), leading me to further inspection of the two subspecies separately. A significant negative correlation between tarsus length and velocity was found in *svecica* (Pearson: $n = 24$, $r = -0.47$, $p = 0.02$), while there was no such relationship in *namnetum* (Pearson: $n = 12$, $r = 0.20$, $p = 0.54$). None of the other morphological characters were significantly related to any of the sperm characters (all $p > 0.17$).

Discussion

I found little evidence for relationships between heterozygosity and any of the sperm characters, but there was a significant relationship between heterozygosity and velocity when including data from all males (i.e. also those with < 10 motile sperm filmed). In support of previous studies, I found a strong genetic differentiation between the two subspecies, as well as differences in individual heterozygosity, in body size and red border width. Furthermore, the two subspecies differed strongly in sperm morphology, the proportion of motile sperm and the coefficient of variation in sperm length within males. In contrast, there was no significant difference between the two subspecies in sperm velocity. There were no significant relationships between sperm morphology and aspects of sperm behavior.

Relationships between genetic diversity and sperm characters

Individual genetic diversity was not related to any of the sperm characters in the two subspecies. This is also true for sperm velocity and proportion of motile sperm, when restricting the analyses to males from which at least 10 individual sperm cells had been measured. This cut-off is commonly used in many studies of passerine species (e.g. Kleven *et al.* 2009, Rowe *et al.* 2013). Hence, based on the most stringent analyses of my data, my hypothesis, which stated that heterozygosity should influence the expression of fitness-related sperm characters positively, should be rejected. The findings in this study are apparently contrary to what Gage *et al.* (2006) found in their study on wild rabbits, and Fitzpatrick and Evans (2009) found in their multiple mammal study. There are several possible explanations for this discrepancy. First, the wild rabbit study of Gage *et al.* (2006) is based on isolated populations spread out in the United Kingdom and associated islands. Island populations are known for having higher risk of genetic depletion through inbreeding and hence increased homozygosity (Frankham 1997). In addition, Fitzpatrick and Evans (2009) only found such a relationship in endangered species, which again implicate small populations and smaller gene pools, which may also lead to increased homozygosity. As far as I know, there are no known inbreeding problems in the bluethroat populations. Nevertheless, *namnetum* is confined to small, somewhat isolated populations, and may hence be under influence of founder effects (genetic bottleneck) (Charlesworth *et al.* 2003) and inbreeding. Therefore, there were reasons to expect stronger relationships between variation in heterozygosity and sperm characters in *namnetum*. However, even if *namnetum* did show higher levels of homozygosity than *svecica*,

I found no greater effects on sperm characteristics in this population. It is possible that although *namnetum* is a smaller population, it has not reached a critical level of homozygosity, with associated effects on sperm characteristics. Additionally, the smaller sample size from *namnetum* and hence lower statistical power, may have counteracted any increased likelihood of finding a significant effect due to increased level homozygosity. Second, even if there was little evidence for relationships with overall heterozygosity, there could be local effects between individual markers in linkage disequilibrium with functional loci with a fitness effect (Hansson *et al.* 2004). This should be followed up by further tests of the effects of single markers. Finally, when including all males, with 4 or more motile sperm cells, I found a significant positive relationship between heterozygosity and sperm velocity, and a similar trend between heterozygosity and the proportion of motile sperm cells. Even if I cannot exclude the possibility that including males with low number of sperm measured introduced an unknown bias in the data, it is also possible that the lack of correlation between heterozygosity and sperm velocity in the most stringent dataset is due to small sample size causing a lower statistical power. There is clearly a need for further studies with larger sample sizes before a firm conclusion can be reached regarding the relationship between heterozygosity and sperm characters in the bluethroat.

Between-population differences in genotypes and phenotypes

The results from this study clearly support the earlier findings of the two subspecies` distinctness, in genetic constitution, body size and sperm characters. Johnsen *et al.* (2006) found that 8 per cent of the variation in microsatellite allele frequencies resided between *namnetum* and *svecica*, while I found a similar percentage (6.6 per cent) in my dataset, which is bigger, both in terms of number of specimens and microsatellite loci. All together these two studies reach the same conclusion; *namnetum* and *svecica* are two genetically distinct subspecies of bluethroat. Another study by Questiau *et al.* (1998), using mitochondrial DNA (mtDNA) markers (control region and cytochrome b gene), also found support for genetic distinctness of these subspecies. In contrast, Zink *et al.* (2003) concluded that there was no support for subspecies based on mtDNA. Similar to Hogner *et al.* (2013), I found differentiation in sperm morphology components between *namnetum* and *svecica*. Moreover, due to an increase in sample size, differences were more evident in the current study, with significant differences in all the components (except: tail length, see Table 1 above), while in Hogner *et al.* (2013) only sperm head length was significantly different. The two populations

differed significantly in heterozygosity, were *namnetum* was more homozygous than *svecica*. The within-male coefficient of variation was significantly different in the two populations, with more sperm length variation within *namnetum* than *svecica* (see Table 1). One may speculate whether there might be a connection between the level of heterozygosity and within-male coefficient of variation in sperm length at the population level (higher level of homozygosity being associated with higher level of sperm variation within males). However, it should be noted that there was no such relationships at the level of individual males within each of the two populations.

There are significant differences between *namnetum* and *svecica* in all of the body morphological measurements (tarsus length, wing length and red border width) in this study. Interestingly red border was wider in *namnetum* than in *svecica*, and with a lower variance. This may be an indication of stronger selection on this secondary sexual trait in *namnetum* than in *svecica* (Andersson 1994).

Relationships between sperm morphology and sperm behavior

I did not find any relationship between total sperm length and velocity. A similar lack of relationship between sperm length and velocity has been reported in other studies, e.g. on tree swallows (*Tachycineta bicolor*) (Laskemoen *et al.* 2010) and four species of new world blackbirds (Lüpold *et al.* 2009b). On the other hand, a study of the sand martin (*Riparia riparia*) reported a negative association between sperm length and velocity, but longer sperm had higher longevity than shorter sperm (Helfenstein *et al.* 2008), and a study of pied flycatchers (*Ficedula hypoleuca*) found that longer sperm had a greater reduction in sperm velocity than shorter sperm over a time frame of 10 minutes after sampling (Lifjeld *et al.* 2012). In contrast, in zebra finches (*Taenopygia guttata*) Mossman *et al.* (2009) found positive associations between sperm length and velocity. Additionally, a study on multiple passerine species found no relationship between the sperm length and velocity (Kleven *et al.* 2009), whereas another study found positive correlations, in both a wide range of passerine species and among closely related species of a single family (*Icteridae*) (Lüpold *et al.* 2009a). These case studies and comparative analyses exemplify the lack of consensus as to how sperm swimming speed changes as a function of sperm length both within and among species.

I found no relationship between the within-male coefficient of variation in sperm length and sperm velocity in any of the two subspecies, which may indicate that sperm uniformity do not

influence the velocity in bluethroats. Coefficient of variation within male is lower than coefficient of variation between males in both subspecies (see Table 1 above), which indicates more sperm variation between males than within the male (Laskemoen *et al.* 2007). A recent study concluded that the between-male coefficient of variation in sperm length has a great potential as an index of sperm competition in comparative analysis of passerine birds (Lifjeld *et al.* 2010). The between-male coefficient of variation is quite similar in the two subspecies, which further supports that the level of sperm competition is relatively similar in the two subspecies (Questiau *et al.* 1999, Johnsen and Lifjeld 2003, Hogner *et al.* 2013). There was a significant negative correlation between within-male coefficient of variation and midpiece/total sperm length ratio in *svecica*, and a trend in *namnetum*. This ratio is often referred to as a proxy of the sperm cell's mitochondria (i.e. the cell's energy) (e.g. Rowe *et al.* 2013), which here would be indicating that the more variation within the male, all the lower energy the sperm cell holds. However, it is more likely that these two variables are influenced by a third common factor, for example general condition and/or the degree of developmental stability.

Relationships between biometry and sperm behavior

There was a significant negative correlation between tarsus length and velocity in *svecica*, but not in *namnetum*. This result is similar to the findings by Helfenstein *et al.* (2008), who found that smaller males (estimated by body mass) of sand martins (*Riparia riparia*) had faster swimming sperm, but shorter longevity, than bigger males. Hence, bigger males may have a slower swimming sperm, but possibly sperm that are able to swim for longer. Longevity was not measured in this study, and the surprising negative relationship between male size and sperm velocity clearly needs further investigation.

Conclusion

I found little evidence for the hypothesis that individual heterozygosity positively influences the expression of fitness-related sperm characteristics. However, there was a significant relationship in the predicted direction between heterozygosity and velocity when including data from all males. Further studies with larger sample sizes both with respect to sperm and genetics are needed to reach a firm conclusion regarding the relationship between heterozygosity and sperm characters in the bluethroat.

References

Andersson, M. (1994). "Sexual selection." Princeton, *Princeton Univerity Press*.

Balshine, S., Leach, B. J., Neat, F., Werner, N. Y. and Montgomerie, R. (2001). "Sperm size of African cichlids in relation to sperm competition." *Behavioral Ecology* **12**(6): 726-731.

Breed, W. G. and Taylor, J. (2000). "Body mass, testes mass, and sperm size in murine rodents." *Journal of Mammalogy* **81**(3): 758-768.

Charlesworth, B., Charlesworth, D. and Barton, N. H. (2003). "The effects of genetic and geographic structure on neutral variation." *Annual Review of Ecology Evolution and Systematics* **34**: 99-125.

Charlesworth, D. and Willis, J. H. (2009). "The genetics of inbreeding depression." *Nature Reviews Genetics* **10**(11): 783-796.

Cohen, J. (1977). "Reproduction." London, *Butterworths*.

Coltman, D. W., Pilkington, J. G., Smith, J. A. and Pemberton, J. M. (1999). "Parasite-mediated selection against inbred Soay sheep in a free-living, island population." *Evolution* **53**(4): 1259-1267.

Cramp, S. (1988). "The Birds of the Western Palaearctic - Tyrant Flycatchers to Thrushes Vol 5." Oxford *Oxford University Press*.

Fitzpatrick, J. L. and Evans, J. P. (2009). "Reduced heterozygosity impairs sperm quality in endangered mammals." *Biology Letters* **5**(3): 320-323.

Foerster, K., Delhey, K., Johnsen, A., Lifjeld, J. T. and Kempenaers, B. (2003). "Females increase offspring heterozygosity and fitness through extra-pair matings." *Nature* **425**(6959): 714-717.

Frankham, R. (1997). "Do island populations have less genetic variation than mainland populations?" *Heredity* **78**: 311-327.

Gage, M. J. G. (1994). "Associations between body-size, mating pattern, testis size and sperm lengths across butterflies." *Proceedings of the Royal Society B-Biological Sciences* **258**(1353): 247-254.

Gage, M. J. G. and Freckleton, R. P. (2003). "Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length." *Proceedings of the Royal Society B-Biological Sciences* **270**(1515): 625-632.

Gage, M. J. G., Surridge, A. K., Tomkins, J. L., Green, E., Wiskin, L., Bell, D. J. and Hewitt, G. M. (2006). "Reduced heterozygosity depresses sperm quality in wild rabbits, *Oryctolagus cuniculus*." *Current Biology* **16**(6): 612-617.

Goudet, J. (1995). "FSTAT (Version 1.2): A computer program to calculate F-statistics." *Journal of Heredity* **86**(6): 485-486.

Hansson, B. and Westerberg, L. (2002). "On the correlation between heterozygosity and fitness in natural populations." *Molecular Ecology* **11**(12): 2467-2474.

Hansson, B., Westerdahl, H., Hasselquist, D., Akesson, M. and Bensch, S. (2004). "Does linkage disequilibrium generate heterozygosity-fitness correlations in great reed warblers?" *Evolution* **58**(4): 870-879.

Helfenstein, F., Szep, T., Nagy, Z., Kempenaers, B. and Wagner, R. H. (2008). "Between-male variation in sperm size, velocity and longevity in sand martins *Riparia riparia*." *Journal of Avian Biology* **39**(6): 647-652.

Herdegen, M., Nadachowska-Brzyska, K., Konowalik, A., Babik, W. and Radwan, J. (2013). "Heterozygosity, sexual ornament and body size in the crested newt." *Journal of Zoology* **291**(2): 146-153.

Hogner, S., Laskemoen, T., Lifjeld, J. T., Pavel, V., Chutny, B., Garcia, J., Eybert, M. C., Matsyna, E. and Johnsen, A. (2013). "Rapid sperm evolution in the bluethroat (*Luscinia svecica*) subspecies complex." *Behavioral Ecology and Sociobiology* **67**(8): 1205-1217.

Hosken, D. J. (1997). "Sperm competition in bats." *Proceedings of the Royal Society B-Biological Sciences* **264**(1380): 385-392.

Immler, S. and Birkhead, T. R. (2007). "Sperm competition and sperm midpiece size: no consistent pattern in passerine birds." *Proceedings of the Royal Society B-Biological Sciences* **274**(1609): 561-568.

Johnsen, A., Andersson, S., Fernandez, J. G., Kempenaers, B., Pavel, V., Questiau, S., Raess, M., Rindal, E. and Lifjeld, J. T. (2006). "Molecular and phenotypic divergence in the bluethroat (*Luscinia svecica*) subspecies complex." *Molecular Ecology* **15**(13): 4033-4047.

Johnsen, A., Andersson, S., Ornborg, J. and Lifjeld, J. T. (1998a). "Ultraviolet plumage ornamentation affects social mate choice and sperm competition in bluethroats (Aves : *Luscinia s. svecica*): a field experiment." *Proceedings of the Royal Society B-Biological Sciences* **265**(1403): 1313-1318.

Johnsen, A. and Lifjeld, J. T. (1995). "Unattractive males guard their mates more closely - an experiment with bluethroats (Aves, Turdidae: *Luscinia s. svecica*)." *Ethology* **101**(3): 200-212.

Johnsen, A. and Lifjeld, J. T. (2003). "Ecological constraints on extra-pair paternity in the bluethroat." *Oecologia* **136**(3): 476-483.

Johnsen, A., Lifjeld, J. T., Rohde, P. A., Primmer, C. R. and Ellegren, H. (1998b). "Sexual conflict over fertilizations: female bluethroats escape male paternity guards." *Behavioral Ecology and Sociobiology* **43**(6): 401-408.

Kempenaers, B. (2007). "Mate choice and genetic quality: A review of the heterozygosity theory". *Advances in the Study of Behavior*. H. J. Brockmann, T. J. Roper, M. Naguib et al. **37:** 189-278.

Kleven, O., Fossøy, F., Laskemoen, T., Robertson, R. J., Rudolfsen, G. and Lifjeld, J. T. (2009). "Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in passerine birds." *Evolution* **63**(9): 2466-2473.

Kleven, O., Laskemoen, T., Fossøy, F., Robertson, R. J. and Lifjeld, J. T. (2008). "Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds." *Evolution* **62**(2): 494-499.

Laskemoen, T., Kleven, O., Fossøy, F. and Lifjeld, J. T. (2007). "Intraspecific variation in sperm length in two passerine species, the Bluethroat *Luscinia svecica* and the Willow Warbler *Phylloscopus trochilus*." *Ornis Fennica* **84**(3): 131-139.

Laskemoen, T., Kleven, O., Fossøy, F., Robertson, R. J., Rudolfsen, G. and Lifjeld, J. T. (2010). "Sperm quantity and quality effects on fertilization success in a highly promiscuous passerine, the tree swallow *Tachycineta bicolor*." *Behavioral Ecology and Sociobiology* **64**(9): 1473-1483.

Laskemoen, T., Kleven, O., Johannessen, L. E., Fossøy, F., Robertson, R. J. and Lifjeld, J. T. (2013b). "Repeatability of sperm size and motility within and between seasons in the Barn Swallow (*Hirundo rustica*)." *Journal of Ornithology* **154**(4): 955-963.

Leder, E. H., Karaiskou, N. and Primmer, C. R. (2008). "Seventy new microsatellites for the pied flycatcher, *Ficedula hypoleuca* and amplification in other passerine birds." *Molecular Ecology Resources* **8**(4): 874-880.

Lifjeld, J. T., Laskemoen, T., Kleven, O., Albrecht, T. and Robertson, R. J. (2010). "Sperm length variation as a predictor of extrapair paternity in passerine birds." *Plos One* **5**(10): 8.

Lifjeld, J. T., Laskemoen, T., Kleven, O., Pedersen, A. T. M., Lampe, H. M., Rudolfsen, G., Schmoll, T. and Slagsvold, T. (2012). "No evidence for pre-copulatory sexual selection on sperm length in a passerine bird." *Plos One* **7**(2): 5.

Lüpold, S., Calhim, S., Immler, S. and Birkhead, T. R. (2009a). "Sperm morphology and sperm velocity in passerine birds." *Proceedings of the Royal Society B-Biological Sciences* **276**(1659): 1175-1181.

Lüpold, S., Linz, G. M. and Birkhead, T. R. (2009b). "Sperm design and variation in the New World blackbirds (Icteridae)." *Behavioral Ecology and Sociobiology* **63**(6): 899-909.

Morrow, E. H. and Gage, M. J. G. (2000). "The evolution of sperm length in moths." *Proceedings of the Royal Society B-Biological Sciences* **267**(1440): 307-313.

Mossman, J., Slate, J., Humphries, S. and Birkhead, T. (2009). "Sperm morphology and velocity are genetically codetermined in the zebra finch." *Evolution* **63**(10): 2730-2737.

Møller, A. P. (1997). "Developmental stability and fitness: A review." *American Naturalist* **149**(5): 916-932.

Olano-Marin, J., Mueller, J. C. and Kempenaers, B. (2011a). "Heterozygosity and survival in blue tits (*Cyanistes caeruleus*): contrasting effects of presumably functional and neutral loci." *Molecular Ecology* **20**(19): 4028-4041.

Olano-Marin, J., Mueller, J. C. and Kempenaers, B. (2011b). "Correlations between heterozygosity and reproductive success in the blue tit (*Cyanistes caeruleus*): an analysis of inbreeding and single locus effects." *Evolution* **65**(11): 3175-3194.

Parker, G. A. (1998). "Sperm competition and the evolution of ejaculates: towards a theory base". *Sperm competition and sexual selection*. T. R. Birkhead and A. P. Møller. (eds.) San Diego, Academic Press**:** 3-49.

Perez-Gonzalez, J., Carranza, J., Torres-Porras, J. and Fernandez-Garcia, J. L. (2010). "Low heterozygosity at microsatellite markers in Iberian red deer with small antlers." *Journal of Heredity* **101**(5): 553-561.

Questiau, S., Eybert, M. C., Gaginskaya, A. R., Gielly, L. and Taberlet, P. (1998). "Recent divergence between two morphologically differentiated subspecies of bluethroat (Aves : Muscicapidae : *Luscinia svecica*) inferred from mitochondrial DNA sequence variation." *Molecular Ecology* **7**(2): 239-245.

Questiau, S., Eybert, M. C. and Taberlet, P. (1999). "Amplified fragment length polymorphism (AFLP) markers reveal extra-pair parentage in a bird species: the bluethroat (*Luscinia svecica*)." *Molecular Ecology* **8**(8): 1331-1339.

Rowe, M., Laskemoen, T., Johnsen, A. and Lifjeld, J. T. (2013). "Evolution of sperm structure and energetics in passerine birds." *Proceedings of the Royal Society B-Biological Sciences* **280**(1753): 9.

Stockley, P., Gage, M. J. G., Parker, G. A. and Møller, A. P. (1997). "Sperm competition in fishes: The evolution of testis size and ejaculate characteristics." *American Naturalist* **149**(5): 933-954.

Svensson, L. (1992). "Identification guide to European passerines." Stockholm, *British Trust for Ornithology*.

Weir, B. S. and Cockerham, C. C. (1984). "Estimating F-statistics for the analysis of population structure." *Evolution* **38**(6): 1358-1370.

Wolfson (1952). "Breeding Condition in Male Passerine." *Bird-Banding* **23**: 159-165.

Zink, R. M., Drovetski, S. V., Questiau, S., Fadeev, I. V., Nesterov, E. V., Westberg, M. C. and Rohwer, S. (2003). "Recent evolutionary history of the bluethroat (*Luscinia svecica*) across Eurasia." *Molecular Ecology* **12**(11): 3069-3075.

Appendix

Table 2: An overview of the panels, including information of dye type, range size, volume of each primer used (include both forward and reverse), annealing temperature for each marker.

Microsatellites / Markers	Dye	Range	Panel	Volume of each primer to primer mix	Volume for primer mix	PCR annealing temperature
EST10	PET	130-175	1	1.5µl	100µl	59
ZF-C59	PET	280-320	1	8µl	100µl	59
EST9	VIC	375-455	1	1.5µl	100µl	59
GG-C25	FAM	225-265	1	1.5µl	100µl	59
EST31	FAM	325-360	1	5µl	100µl	59
ZF-S8	NED	225-265	1	1.5µl	100µl	59
ZF-S9	NED	135-185	1	6µl	100µl	59
EST16	NED	280-320	1	4µl	100µl	59
EST46	NED	200-250	2	2µl	100µl	56
EST62	VIC	385-430	2	3µl	100µl	56
Fh310	PET	280-340	2	2µl	100µl	56
Fh326	NED	320-350	2	3µl	100µl	56
Fh336	PET	125-205	2	3µl	100µl	56
FH350	NED	100-140	2	3µl	100µl	56
FH361	NED	370-400	2	3µl	100µl	56
FH407	FAM	170-250	2	3µl	100µl	56
FH413	VIC	370-570	3	4.5µl	150µl	56
FH304	VIC	200-280	3	6µl	150µl	56
FH408	NED	115-290	3	6µl	150µl	56
FH403	FAM	100-190	3	4.5µl	150µl	56
FH405	PET	95-200	3	12µl	150µl	56
FH306	FAM	380-515	3	12µl	150µl	56
FH356	PET	350-390	3	3µl	150µl	56
Fh344	VIC	300-330	3	6µl	150µl	56
Fh354	FAM	335-400	4	1.5µl	150µl	56
Fh431	NED	165-220	4	3µl	150µl	56
Fh448	PET	110-200	4	3µl	150µl	56
Fh452	VIC	265-320	4	3µl	150µl	56
Fh465*	**FAM**	**165-280**	**4**	**1.5µl**	**150µl**	**56**
Fh466	VIC	125-175	4	6µl	150µl	56
EST-17	NED	280-430	4	4.5µl	150µl	56
Fh221	VIC	140-190	5	4.5µl	150µl	56
Fh227	PET	215-250	5	4.5µl	150µl	56
Fh224	NED	320-405	5	6µl	150µl	56
Fh225	PET	340-390	5	4.5µl	150µl	56
Fh230	VIC	330-390	5	7.5µl	150µl	56
Fh359	FAM	190-240	5	4.5µl	150µl	56

*Marker that did not give any result. Therefore taken out from further analyses.

Table 3: The characteristics of the microsatellites I used. k is the number of alleles, N is the number of individuals, H(obs.) is the observed heterozygosity, H(exp.) is the expected heterozygosity, PIC is the mean polymorphic information content, HW are the test of Hardy-Weinberg equilibrium (NS: not significant, ND: not done, significance (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$)) and F(null) is the estimate of proportion of null alleles.

Locus	k	N	H(obs.)	H(exp.)	PIC	HW	F(null)
EST10	7	85	0.588	0.688	0.656	NS	+0.0806
ZF-C59	9	89	0.449	0.519	0.482	NS	+0.0761
EST9	29	88	0.909	0.922	0.912	ND	+0.0050
GG-C25	12	91	0.769	0.795	0.759	NS	+0.0117
EST31	9	79	0.380	0.447	0.417	NS	+0.0783
ZF-S8	6	91	0.626	0.609	0.542	NS	-0.0161
ZF-S9	11	86	0.791	0.868	0.847	ND	+0.0408
EST16[1]	**7**	**87**	**0.448**	**0.497**	**0.457**	**NS**	**+0.0555**
EST46	10	92	0.707	0.786	0.752	NS	+0.0537
EST62	8	92	0.641	0.668	0.615	NS	+0.0161
Fh310	17	90	0.778	0.864	0.845	NS	+0.0493
Fh326[2]	**8**	**90**	**0.500**	**0.708**	**0.656**	*******	**+0.1726**
Fh336[2]	**15**	**91**	**0.560**	**0.833**	**0.807**	*******	**+0.1994**
Fh350	5	89	0.191	0.180	0.174	ND	-0.0414
Fh361[2]	**4**	**90**	**0.200**	**0.249**	**0.231**	**ND**	**+0.1131**
Fh407	21	92	0.935	0.895	0.881	ND	-0.0275
Fh413[2]	**3**	**92**	**0.293**	**0.465**	**0.410**	*****	**+0.2294**
Fh304[2]	**23**	**88**	**0.432**	**0.924**	**0.914**	**ND**	**+0.3603**
Fh408	31	87	0.920	0.945	0.937	ND	+0.0107
Fh403	16	92	0.707	0.747	0.716	NS	+0.0264
Fh405[2]	**16**	**85**	**0.435**	**0.892**	**0.876**	**ND**	**+0.3396**
Fh356	3	92	0.130	0.144	0.137	ND	+0.0393
Fh344	7	90	0.600	0.729	0.688	NS	+0.0882
Fh431	8	89	0.629	0.664	0.601	NS	+0.0219
Fh448	20	91	0.791	0.919	0.907	ND	+0.0720
Fh452	6	90	0.389	0.450	0.408	NS	+0.0877
Fh466	8	84	0.726	0.761	0.718	NS	+0.0160
EST-17	13	87	0.747	0.807	0.781	NS	+0.0465
Fh221[2]	**14**	**89**	**0.551**	**0.802**	**0.770**	******	**+0.1844**
Fh227	8	91	0.538	0.582	0.505	NS	+0.0421
Fh224[2]	**25**	**86**	**0.442**	**0.918**	**0.907**	**ND**	**+0.3485**
Fh225	25	89	0.764	0.912	0.900	ND	+0.0864
Fh230	11	91	0.813	0.767	0.739	NS	-0.0391
Fh359	16	91	0.769	0.868	0.852	NS	+0.0627

[1] This showed a linkage to the marker ZF-C59, and was excluded from further analyses.

[2] These markers have a high F(null)-value (> 0.1), and were excluded from further analyses.

Table 4: Overview of allelic richness for the different markers used. The means for the *svecica* and *namnetum* populations are shown separately.

Locus	*svecica*	*namnetum*
EST10	6.79	5.79
ZF-C59	7.25	3.00
EST9	20.38	11.78
GG-C25	8.42	5.74
EST31	5.95	4.00
ZF-S8	5.20	3.00
ZF-S9	8.91	9.64
EST46	8.94	4.92
EST62	6.96	3.72
Fh310	12.80	11.99
Fh350	3.90	3.15
Fh407	16.41	9.54
Fh408	20.25	16.20
Fh403	11.73	7.08
Fh356	2.53	1.98
Fh344	6.65	5.72
Fh431	7.40	3.00
Fh448	17.11	8.60
Fh452	4.38	3.75
Fh466	6.95	4.90
EST-17	10.44	5.76
Fh227	6.77	3.00
Fh225	17.64	8.65
Fh230	9.71	6.00
Fh359	13.97	8.26